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Abstract: PURPOSE Open fetal spina bifida repair has become a novel clinical standard of care. In very large spina bifida lesions, the skin defect cannot be covered primarily, asking for alternative solutions. We hypothesize that amniotic fluid stem cells (AFSC) could be differentiated into keratinocytes that could then be used to bioengineer autologous skin usable for in utero back coverage. METHODS To obtain human AFSC, amniotic fluid samples obtained from fetal surgeries were subjected to immunoselection for c-kit. C-kit-positive samples and controls were cultured with the additives morphogenetic protein 4 and vitamin C to induce differentiation towards keratinocytes. This process was monitored by measuring the expression of K8 and K14 via immunohistochemical staining, flow cytometry, and polymerase chain reaction. RESULTS After immunoselection and expansion, most cells were positive for K8, but none for K14. After completion of the differentiation protocol, cell colonies with keratinocyte-like appearance could be observed, but cells remained positive for K8 and negative for K14, indicating failed differentiation into keratinocytes. CONCLUSIONS Culturing of keratinocyte-like cells from AFSC, harvested intraoperatively, was not feasible in this setting. The reasons for failure must be investigated and eliminated, as bioengineering of fetal skin for clinical use during fetal surgery for spina bifida remains an attractive goal.

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Bioengineering of Fetal Skin: Differentiation of Amniotic Fluid Stem Cells into Keratinocytes

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Keywords
Human spina bifida · Myelomeningocele · Amniocytes · Stem cells · Fetal surgery · Tissue engineering · Amniotic fluid · Keratinocytes

Abstract
Purpose: Open fetal spina bifida repair has become a novel clinical standard of care. In very large spina bifida lesions, the skin defect cannot be covered primarily, asking for alternative solutions. We hypothesize that amniotic fluid stem cells (AFSC) could be differentiated into keratinocytes that could then be used to bioengineer autologous skin usable for in utero back coverage. ***Methods:*** To obtain human AFSC, amniotic fluid samples obtained from fetal surgeries were subjected to immunoselection for c-kit. C-kit-positive samples and controls were cultured with the additives morphogenetic protein 4 and vitamin C to induce differentiation towards keratinocytes. This process was monitored by measuring the expression of K8 and K14 via immunohistochemical staining, flow cytometry, and polymerase chain reaction. ***Results:*** After immunoselection and expansion, most cells were positive for K8, but none for K14. After completion of the differentiation protocol, cell colonies with keratinocyte-like appearance could be observed, but cells remained positive for K8 and negative for K14, indicating failed differentiation into keratinocytes. ***Conclusions:*** Culturing of keratinocyte-like cells from AFSC, harvested intraoperatively, was not feasible in this setting. The reasons for failure must be investigated and eliminated, as bioengineering of fetal skin for clinical use during fetal surgery for spina bifida remains an attractive goal.
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Introduction

Open spina bifida (OSB) is a frequent congenital malformation that causes a disastrous and irreversible cluster of neurological impairments in affected individuals. Anatomically, OSB is characterized by a localized closure defect of the developing spinal cord (non-neurulation), meninges, vertebral arches, overlaying muscles, and skin. Thus, the non-neurulated spinal cord is openly exposed

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to the amniotic fluid and uterine wall. Studies have shown that especially in the third trimester of gestation, the unprotected spinal cord is progressively injured by mechanical trauma and by toxic effects of the amniotic fluid ("two-hit hypothesis") [1]. Early protection of exposed neural tissue by fetal surgery was shown to prevent this injury in experimental studies [2], and finally, a prospective randomized clinical trial (MOMS trial [3]) demonstrated significantly better results in infants after fetal than after postnatal repair. Fetal OSB repair has therefore become a new clinical standard of care for select human fetuses suffering from this devastating malformation.

Within the phenotypic spectrum of OSB, some lesions are very large, and, consequently, the skin defect cannot be primarily closed, requiring alternative approaches. In this context, we have pioneered the use of biosynthetic skin substitutes [4] and also the in utero construction of transposition flaps [5], both of which are tenable ways to go. Yet, disadvantages include a necessity for postnatal skin grafting or formal flap closure of donor sites.

In search of potentially better solutions, we hypothesized that bioengineering and in utero application of fetal skin might be an elegant solution.

Our research group has already conducted various experiments to engineer and transplant fetal dermoepidermal skin substitutes (fDESS): We showed that using small fetal skin biopsies, fDESS could be successfully bioengineered in the laboratory [6] and grafted in vivo onto immunoincompetent rats [7]. Lastly, we successfully transplanted autologous fDESS in utero onto sheep fetuses (manuscript in preparation). Of note, a first step towards tissue engineering of a fDESS without the need of a skin biopsy was published by our group earlier, showing that amniotic fluid mesenchymal cells could be used instead of fibroblasts to build the dermal part of a fDESS [8].

Here, we report on our attempt to stimulate amniotic fluid stem cells (AFSC) to differentiate into keratinocytes (KC) that could then be used to bioengineer fetal skin for eventual clinical application.

Materials and Methods

Human amniotic fluid samples were obtained during human fetal surgeries for OSB right after opening the uterus. Care was taken to minimize sample contamination by maternal blood. We used amniotic fluid samples from 5 fetal surgeries (gestational ages between 24 and 25 weeks). All amniotic fluid samples were expanded on glass cover slides for 10 days in Chang B+C medium® (Irvine Scientific). Selection of AFSC and differentiation into KC were done as described by Sun et al. [9]. Briefly, once growing of mesenchymal appearing cell colonies was observed, immunoselection for c-kit with the MACS system (Miltenyi Biotec) and subsequent expansion in nontreated 24-well multidishes in the Chang complete medium® (MEM Alpha containing 20% Chang B+C, 15% fetal bovine serum, penicillin, and streptomycin [5 per 500 mL medium] and L-glutamine [2 mM final]) was performed. Cells were checked daily for morphology, and the medium was changed three times per week. When cells reached 70% confluence, they were trypsinized and seeded onto uncoated 35-mm dishes. Cells were transferred to 6-well culture dishes at a seeding density of 2×10^4 cells per well after passage 3 and were differentiated in KBM™-2 Basal Medium (Lonza) with additional supplements (10% fetal bovine serum, 10 ng/mL recombinant human EGF [R&D Systems, Minneapolis, MN, USA], 0.5 nM human recombinant BPM-4 [R&D Systems], 50 µg/mL ascorbic acid [Sigma-Aldrich], 5 µg/mL insulin [Sigma-Aldrich], 0.5 µg/mL hydrocortisone [Sigma-Aldrich], 0.1 nM cholera toxin [Sigma-Aldrich], 1.37 ng/mL triiodothyronine [Sigma-Aldrich], 24 µg/mL adenine [Sigma-Aldrich]), and KC-conditioned medium in a 1:1 ratio for 30 days.

For control, samples of c-kit-negative fractions and samples without immunoselection were cultured analogously.

Monitoring

Cells were monitored at regular time intervals for keratin 8 and keratin 14 expression. The percentage of cells that express K8 or K14, respectively, was determined either by flow cytometry or by immunohistochemistry (K8: clone K8.8, 1:100, K14: clone EP1612Y1:500; Abcam), which also allowed to observe cell morphology. Nuclei were stained with 1 µg/mL Hoechst 33341 (Sigma). As positive controls for K14, either HaCaT cells or human KC from full skin biopsies were used. For polymerase chain reaction (PCR) analysis, cells of some culture plates were collected in suspension and frozen in liquid nitrogen at different stages of differentiation. PCR was performed simultaneously on all samples using primers for K5/K14 and K8/18.

Results

At the first medium change after 7 days, plates revealed cell colonies of different morphologies, some with mesenchymal and others with epithelial appearance (Fig. 1). Only few cells could be detected microscopically after immunoselection for c-kit with the MACS® system. Cell morphology and composition after expansion of these cells did not show any striking difference compared to c-kit-negative and unselected amniocyte cultures. In addition, the c-kit-positive, c-kit-negative, and unselected amniocyte cultures remained morphologically undistinguishable during the whole differentiation period.

Immunohistochemistry showed that the majority of cells in all cultures were positive for K8 (a marker of simple, not stratified epithelia) (Fig. 2), suggesting optimal preconditions for epithelial differentiation, but no K14-positive cells (K14 is found in basal KC) could be detected.

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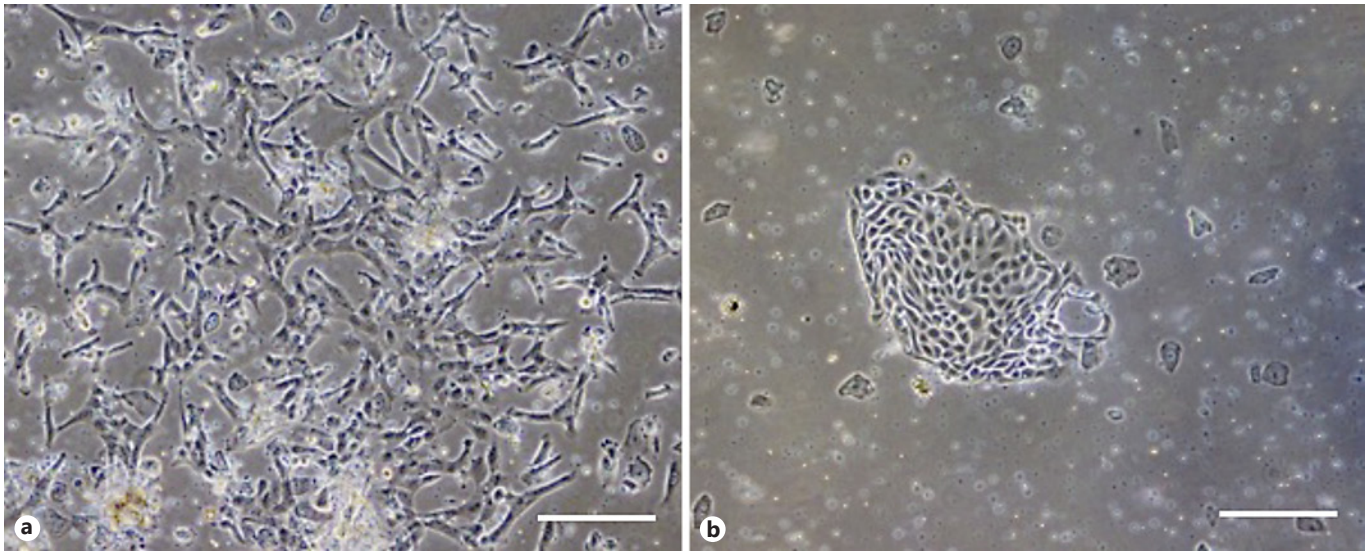

Fig. 1. Mesenchymal (**a**) and epithelial (**b**) appearance of human amniocytes on glass cover slides after 7 days in the preselection medium (Chang B+C Medium). Bars = 100 μm.

Furthermore, after having completed the 30-day differentiation protocol, both, the c-kit-positive and -negative cell fractions were not different in terms of morphology, growth characteristics, or immunohistochemical staining. Several culture plates contained cells with a KC-like morphology, as seen in Figure 3. The c-kit-negative population exhibited a morphology similar to the cobblestone appearance of KC. While most cells were still positive for K8, K14-positive cells were not found.

PCR and flow cytometry results of different samples during the differentiation process yielded identical findings: the amount of K8-positive cells varied; however, keratin14 was not expressed (data not shown).

With increasing passage (up to p10–13), proliferation of cells decreased, and cells started to detach, likely because of senescence.

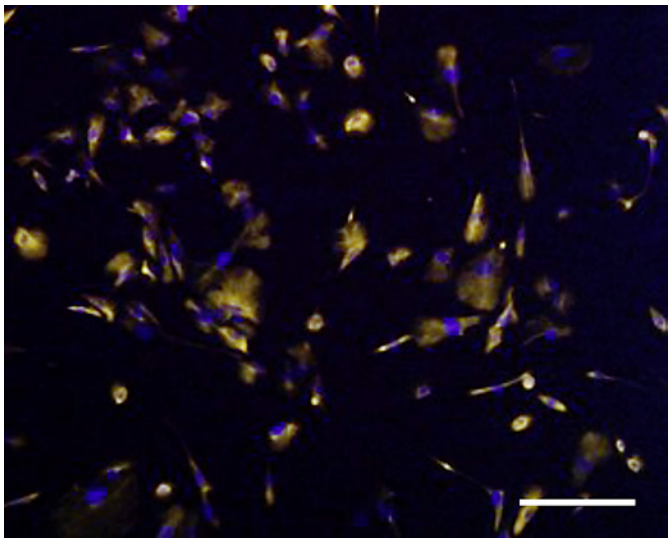

Fig. 2. Immunohistochemistry of amniotic fluid cells staining positive for K8 (orange, Alexa Fluor® 555) during differentiation. These cells were selected for c-kit. Nuclei stained with Hoechst (blue). Bar 100 μm.

Discussion

The hypothesis that amniotic fluid might be a valuable repository for stem cells that could be used to create close to normal autologous fetal skin is highly attractive. This is especially true for the clinical scenario of large defects in fetal spina bifida repair where direct skin closure is not possible and some sort of substitutes are needed. As all patients planned for fetal spina bifida repair do undergo preoperative amniocentesis to exclude genetic anomalies, amniotic fluid would always be available. Therefore, the goal of this study was to differentiate AFSC into KC that could then be used to bioengineer autologous skin for in utero back coverage of large spina bifida defects.

The results of the study show that after immunoselection and completion of the differentiation protocol, we did obtain cell colonies with KC-like appearance in both c-kit-positive and -negative fractions. However, these

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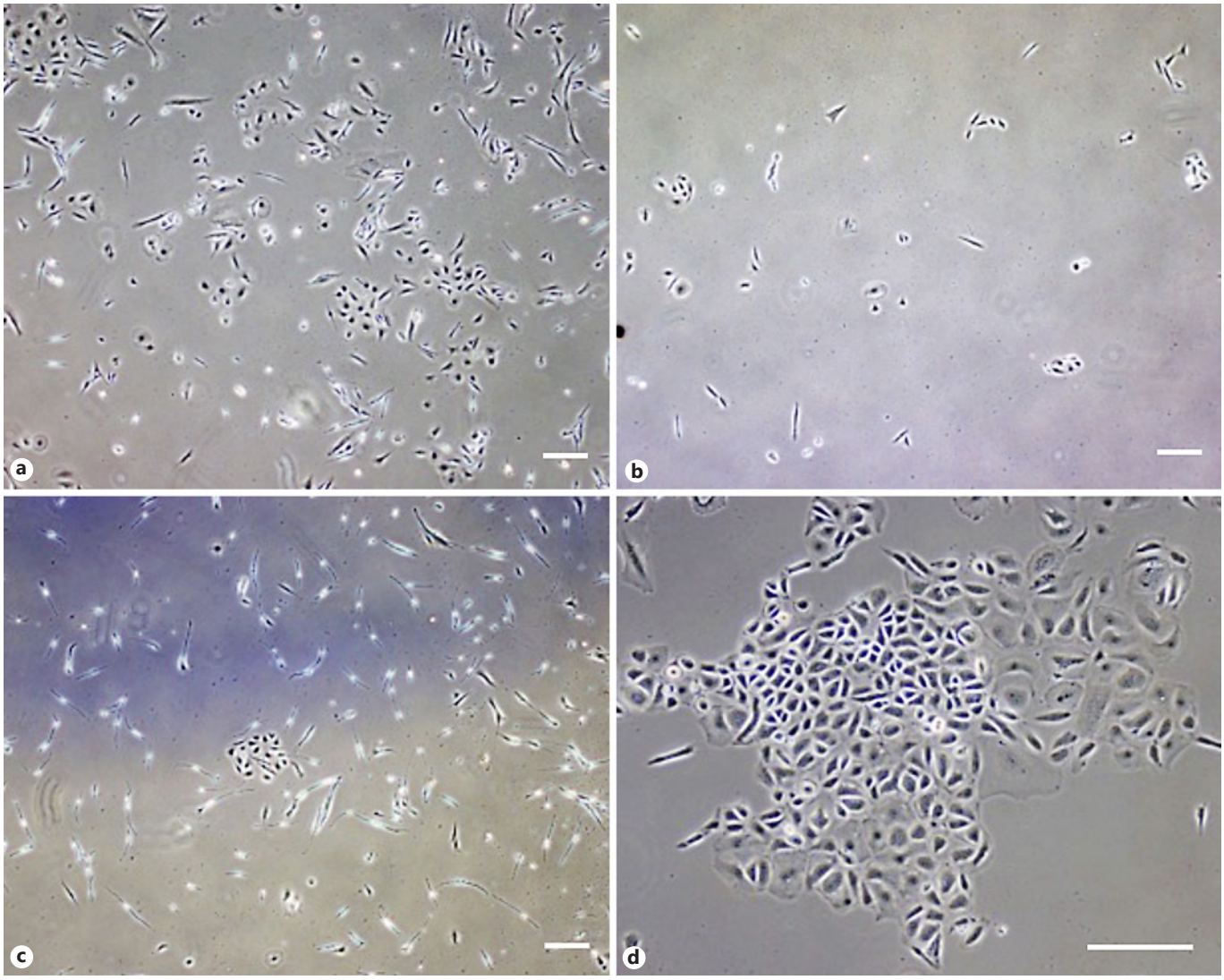

Fig. 3. Different amniocyte fractions after the 30-day differentiation protocol in KGM™-2 (all bars = 100 μm). **a** Amniocytes from the c-kit-negative fraction after 2 weeks in KGM-2 (p1). **b** Amniocytes from the c-kit-positive fraction after 2 weeks in KGM-2 (p2). **c** Amniocytes from samples without c-kit immunoselection after 2 weeks in KGM-2 (p1). **d** Amniocytes from the c-kit-negative fraction after 2.5 weeks in KGM-2 (p3).

cells were simple epithelial cells, as shown by immunostaining (i.e., positive for cytokeratin K8) and they were lost after several passages, most likely due to senescence. Thus, within the particular setting of our experiments, it was not possible to obtain classical KC from AFSC.

Interestingly, other groups have performed similar experiments yet with other goals and were able to show that AFSC can be successfully differentiated into cell lines of all three germ layers [10], and in particular also into KC [9]. As in these quoted studies, our experiments were also based on the same two pillars: the isolation of AFSC by selection for c-kit and the use of bone morphogenetic protein 4 (BMP-4) and ascorbic acid (vitamin C [VitC]) for the differentiation into KC.

De Coppi et al. [10] described the isolation of AFSC using selection for c-kit (CD117), obtaining 1% of cells that were pluripotent stem cells capable of differentiating into lineages of the three embryonic germ layers. AFSC were shown to exhibit intermediate characteristics between embryonic stem cells and mesenchymal stem cells [11]. However, Arnhold et al. [12] compared c-kit-positive cell fractions with c-kit-negative and nonselected populations and found that the neuronal differentiation potential was higher in the c-kit-negative fraction. Al-

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though this was never examined, based on the common neuroectodermal origin of neurons and KC, one could deduce from Arnhold's results that also epidermal differentiation should be higher in the c-kit-negative fraction. We therefore also used the c-kit-negative fraction in our experiments. Sun et al. [9] were the first to describe differentiation of AFSC into KC, isolating AFSC as described by De Coppi [10] with c-kit selection and then using BMP-4 and VitC during the differentiation process.

Movahednia et al. [13] reviewed several studies on the differentiation of embryonic stem cells into KC. Most of these studies used BMP-4, which is an inhibitor of neural differentiation into embryonic stem cells at some point during differentiation. Guenou et al. [14] added ascorbic acid to the use of BMP-4. Ascorbic acid is known to act as a radical scavenger and to promote KC survival [15]. We used both BMP-4 and VitC as described by Sun and applied it to c-kit-positive, c-kit-negative, and unselected cultures. The differentiation progress was monitored by the mean of observing transition of expression of K8/18 to K5/14, as it was described for studies differentiating embryonic stem cells into KC [14] and also by Sun et al. [9]. K8/18 form heteropolymeric intermediate filaments in simple epithelia. They are the first keratins that appear in embryogenesis but disappear in differentiating KC and are normally not present in the stratified epidermis [16]. K5 and K14 are heteropolymers that make up the intracellular cytoskeletal scaffold of the basal KC in a stratified epidermis [17]. Despite adherence to the concepts and protocols of the above-mentioned studies, we could not observe any epidermal differentiation; i.e., the transition of K8/18 expression to K5/14, whether by immunohistochemistry nor flow cytometry and PCR.

In light of the published successful "creation" of ectodermal cells [10] and specifically KC [9], possible causes for failure in our setting were analyzed. One distinct difference between the successful AFSC experiments and our setting was found: the source of amniotic fluid used. In our assays, we used amniotic fluid from open fetal surgeries (access to the uterine cavity by hysterotomy). This source entails several possible pitfalls. First, the amniotic fluid was often contaminated with maternal blood and cell detritus. This hampered the observation of cell morphology during the first 5 days, and we do not know whether cell detritus and blood constituents had a negative influence on AFSC; e.g., by disturbing their attachment. Second, amniotic fluid of a pregnancy with spina bifida might result in a different response of the AFSC to the differentiation protocol. Ceccarelli et al. [18] showed that sheep AFSC in myelomeningocele models exhibit different gene expression patterns and metabolic variations compared to those of healthy fetuses. Third, the amniotic fluid we used was of advanced gestational age (between 24 and 25 weeks). Moschidou et al. [19] reported a higher fraction of pluripotent cells in amniotic fluid of lower gestational age. Prior studies have suggested 16–22 weeks to be the optimal gestational age for good-quality amniotic fluid to isolate c-kit-positive stem cells. The gestational age of these samples varied between 16 and 31 weeks [20]. Similarly, Sun et al. [9] used amniotic fluid samples ranging from 19 to 22 weeks of gestation to differentiate KC from amniotic fluid. One study compared markers of amniotic fluid cells by real-time PCR at weekly time points between 15 and 20 weeks gestational age and found c-kit to disappear at 19–20 weeks [21]. This is lower than the suggested 16–22 weeks for optimal c-kit sampling by a different group [20]. Another study showed that second-trimester amniotic fluid did not reveal any c-kit-positive cells in a fluorescence-activated cell sorting analysis, but many other pluripotency markers [22]. Hence, a limitation of our study was to rely solely on c-kit expression, since it may no longer have been expressed at the gestational age of our amniotic fluid samples.

Unfortunately, we were not able to use amniotic fluid from amniocenteses of earlier gestational ages and from pregnancies without OSB. Due to the increasing use of noninvasive pregnancy testing, amniocentesis in general is performed less often, resulting in sample paucity for research purposes. The preoperative amniocentesis samples from our fetal surgery patients were all used for diagnostic purposes and were not available to us for this experimental line. However, we are convinced that these same experiments must be repeated with both amniocenteses samples from patients carrying a fetus with OSB and from pregnancies with healthy fetuses, obtained earlier in gestation.

Another pertinent point that deserves a comment is whether the duration needed to differentiate KC from AFSC does allow a clinical use for the envisioned scenario. Although OSB is typically diagnosed in the second trimester, in expert hands it can also be reliably detected early in gestation during the 11–14 weeks screening [23, 24]. If amniocentesis is performed at 15 weeks, up to 10 weeks remain for the bioengineering process, as fetal OSB repair is performed at 23–26 weeks. The differentiation process has been reported to last 30 days [9]; thereafter, 5–6 weeks remain for expansion and application in the organotypic cultures. Based on this calculation, the envisioned clinical application seems realistic.

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However, for reasons considered above, particularly the aspects of different gene expression patterns and metabolic variation in AFSC of OSB samples, the envisioned goal may be unreachable. More generally, our findings also prompt interesting new questions: do severe congenital malformations, particularly those with a large abnormal body opening towards the amniotic cavity (e.g., OSB with outflow of fetal cerebrospinal fluid, gastroschisis, and omphalocele) influence AFSC in any significant way?

Last, although the immature immune system of the fetus might tolerate also allogeneic KC obtained from other sources, the readily available amniotic fluid in patients planned to have fetal OSB repair remains an attractive source to investigate on.

Conclusion

Differentiation of AFSC into phenotypically classical KC failed in our setting. The specific amniotic fluid source in our study; i.e., amniotic fluid from fetal surgery, is seen as the most likely reason for failure. Follow-up experiments using autologous amniotic fluid harvested earlier in gestation by amniocentesis may solve the current problem and ultimately allow culturing of a close-to-natural epidermis suitable for clinical application in utero.

Statement of Ethics

Patient informed consent was obtained for the collection of amniotic fluid during open fetal surgery procedures and its experimental use in the laboratory to tissue engineer skin. The study was approved by the Ethical Committee of the Canton of Zurich (KEK-ZH-Nr. 2015-0247).

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.B. designed the study, performed the experiments, analyzed the data, and wrote and reviewed the manuscript. L.P. and T.B. performed the experiments, analyzed the data, and reviewed the manuscript. E.R. and M.M. discussed the data and reviewed the manuscript. L.M. designed the study, discussed the data, and wrote and reviewed the manuscript.

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